Enhanced

The present invention is directed to the enhanced production and secretion of a protein of interest. When discussing expression enhanced has the following meanings. In the case a homologous protein enhanced should be read as expression above normal levels in said host. In the case of a heterologous protein basically any expression is, of course, enhanced.

When discussing resistance to proteolysis, enhanced means that the protein of interest has an increased half-life when compared to an altered form of the protein of interest, e.g., untagged.

Non-Polar

As used herein, the term "non-polar" refers to the amino acid content of the carboxy tail of a protein. For example, when the last five amino acids at the C-terminus are selected from nonpolar amino acid residues (A, V, L, I, P, F, W, M) the tail is considered nonpolar. If one or two of these last five residues are uncharged polar (G, S, T, C, Y, N, Q), the tail would still be considered substantially nonpolar.

In contrast, if one or two of the last two amino acids at the C-terminus is/are charged polar: D or E (negatively charged) or K, R or H (positively charged), the tail would be considered polar, charged and, according to the present invention, this makes the protein resistant against proteolytic degradation by a subclass of proteases that recognize nonpolar C-terminal tails of secreted proteins.

Tag sequence

As used herein, a "tag sequence" or "tag" refers to a short peptide sequence on the carboxy terminus of an expressed protein that effects the proteolysis of the expressed protein. For example, the bacterial tag encoded by *ssrA* is cotranslationally added to truncated polypeptides, thereby targeting these molecules for proteolytic degradation. It is to be understood that in the present invention the addition of a tag serves to signal a decrease in

proteolytic degradation, i.e., enhanced resistance to proteases, of proteins with substantially non-polar carboxy termini.

Preferably the tag is at least one charged amino acid residue. Preferably, the tag comprises two charged amino acid residues. The charged amino acid residue(s) may be positively charged. Alternatively, the charged amino acid residue(s) may be negatively charged.

The tag should be as short as possible, since the tag itself may influence the activity, specificity etc. of the protein product. In general, one can expect that a short tag of two or three amino acids has no or just a minor effect on folding of the protein (and thereby) the activity, specificity, etc. But there is probably no general rule for this, it depends on the nature of the protein/enzyme.

In another preferred embodiment, the tag may be a modified Bacillus SsrA tag. In an especially preferred embodiment the modified tag has the sequence AGKTNSFNQNVAL**DD** (SEQ ID NO: ___) or AGKTNSFNQNVAL**KK** (SEQ ID NO: ___).

Isolated or Purified

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

Native protein or polypeptide

As used herein, the terms "Native protein" or "Native polypeptide" are used interchangeably herein and refer to a protein or polypeptide which has not been modified or altered at the last two amino acid residues located at the carboxy-terminus. In other words, the last two amino acid residues at the carboxy-terminus of the expressed protein or polypeptide are the same as those found in the naturally occurring protein or polypeptide. Other residues within the protein or polypeptide may be altered, modified or mutated.

Heterologous Protein

As used herein, the term "heterologous protein" refers to a protein or

polypeptide that does not naturally occur in a host cell. Examples of heterologous proteins include enzymes such as hydrolases including proteases, cellulases, amylases, other carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phophatases, hormones, growth factors, cytokines, antibodies and the like.

Homologous Protein

The term "homologous protein" refers to a protein or polypeptide native or naturally occurring in a host cell. The invention includes host cells producing the homologous protein via recombinant DNA technology. The present invention encompasses a host cell having a deletion or interruption of the nucleic acid encoding the naturally occurring homologous protein, such as a protease, and having nucleic acid encoding the homologous protein re-introduced in a recombinant form. In another embodiment, the host cell produces the homologous protein.

Nucleic Acid Molecule

The term "nucleic acid molecule" includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein may be produced. The present invention contemplates every possible variant nucleotide sequence, encoding tag sequences, including but not limited to the individual amino acid residues of D, E, K and N, all of which are possible given the degeneracy of the genetic code.

A "heterologous" nucleic acid construct or sequence has a portion of the sequence that is not native to the cell in which it is expressed. Heterologous, with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by